

HISTOLOGICAL, HORMONAL AND METABOLIC RESPONSE TRIGGERED BY *N*-1-NAPHTHYLPHTHALAMIC ACID-INDUCED STEM SWELLING IN *Solidago canadensis* L.

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ABSTRACT

The effect of *N*-1-naphthylphthalamic acid (NPA, 5.0%, w/w in lanolin) on the growth of *Solidago canadensis* (Canadian goldenrod) stem was studied, focusing on histological analyses, comprehensive analyses of phytohormones and polar metabolites. NPA substantially induced stem swelling at and above the application site and stimulated vascular cambium activity around the area of its application. The cambial zone in the swelling part of the stem was twice as wide as that treated with lanolin only (control). The proliferation of cambial cells increased xylem production and, consequently, vascular bundle thickness. A significant enlargement of parenchymatous pith cells and an increased diameter of the pith were also observed. Comprehensive phytohormone analyses revealed that NPA increased the content of indole-3-propionic acid, indole-3-acetic acid, and indole-3-acetyl-aspartic acid in the swelling part of the stem, as well as *trans*-zeatin riboside. These results suggest that NPA-induced stem swelling depends on the dynamics of changes in aux-in and cytokinin metabolites. Furthermore, the contents of monosaccharides (glucose, fructose and galactose) as well as malic, succinic, fumaric acids, cyclitols and quinic acid derivatives increased markedly in the swelling stem. This may indicate that the site of NPA-induced stem swelling is a physiological sink for polar metabolites needed for the growth of this tissue. Thus, it seems that auxins, in interaction with cytokinins, regulate the strength of the sink, controlling the transport of polar metabolites into the swelling part of *S. canadensis* stem.

Keywords: auxin transport inhibitor, Canadian goldenrod, secondary growth, stem swelling, vascular cambium, *N*-1-naphthylphthalamic acid

INTRODUCTION

The perennial herb *Solidago canadensis* L. (Canadian goldenrod, Asteraceae), native to North America, is an invasive plant widely distributed in many regions of Europe, Asia and Australia [Baranová et al. 2022]. From an ecological point of view, the production and propagation of *S. canadensis* biomass should be limited as much as possible [Bielecka et al. 2017].

In modern crop production, the chemical manipulation of the phytohormone system is used to control weeds. In this context, synthetic phytotropins, which interfere with a plant's auxin system, are used as auxinic herbicides. Phytotropins inhibit polar auxin transport by interacting with a regulatory protein of the auxin efflux carrier, leading to the abnormal accumulation of indole-3-acetic acid (IAA) in plant meristems [Johnston et al. 2020]. *N*-1-naphthylphthalamic acid (NPA) was initially developed as a herbicide called Naptalam [Kapusta 1979]. Subsequently, it was shown to be a key inhibitor of directional polar auxin transport in plant tissues due to its binding to PIN auxin transporters [Abas et al. 2021, Johnston et al. 2020, Kong et al. 2022, Teale and Palme 2018]. *Arabidopsis thaliana* has been established as a model for primary research on the secondary growth of shoots, hypocotyls, and roots [Little et al. 2002]. In *A. thaliana*, there is evidence that auxin (IAA) acts as a key organizer of cambial growth and vascular development and plays a role in positional signaling [Fábregas et al. 2015, Friml 2022]. NPA induced stem thickening through auxin accumulation and stimulation of cambium activity [Zhong and Ye 2001, Little et al. 2002, Suer et al. 2011]. Additionally, NPA enhanced fiber development in the middle part of the stems and shortened inflorescences, resulting in the formation of few flowers or no flowers at all [Zhong and Ye 2001]. Moreover, in *A. thaliana*, NPA increased the width of the fascicular xylem and interfascicular extraxylary fibers [Mattsson et al. 1999]. These findings indicate that NPA significantly affects phytohormone dynamics and histological changes in treated plants. However, to date, there is no available information on the effect of NPA on the levels of other phytohormones in the plant stem. Studies evaluating the effect of NPA on the regulation of primary and secondary meristem activity have fo-

cused exclusively on *A. thaliana* and not on perennial plants.

It has been shown that flavonoids can interfere with auxin transport in plant tissues by modulating it through interactions with regulatory mechanisms, and flavonoid biosynthesis determines where and when these interactions take place [Brown et al. 2001, Peer and Murphy 2007]. However, there are no data on the effect of NPA on the content of these compounds in *S. canadensis*.

This paper presents the results of a study on the physiological and metabolic effects of NPA-induced stem swelling of *S. canadensis* and its histological structure. An attempt was also made to clarify the interactions between particular effects of NPA-induced stem swelling.

MATERIAL AND METHODS

Solidago canadensis L. plants naturally growing in Skierniewice (Poland, GPS: 51°57'50.6"N 20°10'15.2"E) with a height of about 110 cm, were used in the experiment on 4 June 2022. At a distance of 8 cm from the apex, the stem was treated with *N*-1-naphthylphthalamic acid (NPA, 5%, w/w in lanolin) around the stem (2–3 mm wide strip). Control plants were treated in the same way but with lanolin only. Twenty plants were used for each treatment. On 14 June stem swelling was measured at the treatment site and at various distances below and above the treatment site. One-centimeter stem sections were taken for histological examination and metabolic analyses from the NPA and control application sites, as well as 1 cm below the treated parts. The experiment was repeated on 11 and 20 June. Part of the stem pieces were frozen, freeze-dried, and powdered for the determination of phytohormones, polar metabolites, phenolic acids, and flavonoids. Twenty plants were used for each experiment. On 17 August, the size of plant shoots was measured and photographed.

For a histological study, stem pieces were fixed in a mixture of chromic acid, acetic acid, and formalin (CrAF) for 48 hours and embedded in paraffin according to the method previously described [Marasek-Ciolakowska et al. 2020]. Longitudinal and cross-sections (10- μ m thick) were cut with a rotary microtome and stained with 1% safranin and 0.5% fast green.

The sections were mounted in Canada balsam and observed under a light microscope with polarization (Eclipse 80i with NIS-Elements BR ver. 4.00 imaging software, Nikon Instruments Inc., Tokyo, Japan). The stem diameter and the size of stem components (cortex, cortex cells, pith, pith cells, vascular tissue, cambium) were determined.

Analyses of phytohormones were carried out according to the method by Dziurka et al. [2022]. Freeze-dried and finely ground plant materials (20 mg) were spiked with a stable-isotope labelled internal-standard mixture and extracted with buffer (methanol/water/formic acid, 15/4/1, v/v/v). Supernatants were collected and evaporated under a nitrogen stream. The remaining suspension was diluted with 3% methanol in 1 M formic acid and cleaned on hybrid SPE columns (BondElut Plexa PCX, Agilent, USA). Analyses were performed in triplicate on a UHPLC apparatus (Agilent Infinity 1260, Agilent, Germany) coupled to a triple quadrupole mass spectrometer MS/MS (6410 Triple Quad LC/MS, Agilent, USA) equipped with electrospray ionization and an Ascentis Express RP-Amide analytical column (2.7 μ m, 2.1 mm \times 150 mm; Supelco, Bellefonte, PA, USA).

The determination of polar metabolites was carried out according to the method described in detail by Szablińska-Piernik and Lahuta [2021]. Briefly, metabolites were extracted from freeze-dried and powdered tissue with a mixture of methanol: water (1:1), containing ribitol as an internal standard. The extraction was repeated four times, and the obtained crude extracts were collected and centrifuged (20,000 *g* at 4°C for 20 min). The supernatant was then extracted with chloroform to remove nonpolar compounds, and the polar fraction was evaporated to dryness in a speed vacuum rotary evaporator. The polar compounds were derivatized with *O*-methoxyamine hydrochloride in pyridine and then with a mixture of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide in pyridine. The derivatives obtained were separated on a ZEBRON ZB-5MSi Guardian capillary column (Phenomenex, USA) in a gas-chromatograph coupled with a quadrupole mass spectrometer (QP-GC-2010, Shimadzu, Japan). Metabolites were identified by comparing retention times, relative retention times, and retention indices,

determined from the saturated hydrocarbons and mass spectra of original standards purchased from Sigma-Aldrich (USA) and from the NIST 05 library (National Institute of Standards and Technology, USA). Polar metabolites were quantified using original standards purchased from Sigma-Aldrich (USA).

The content of phenolic acids and flavonoids was determined according to the method described by Dębski et al. [2021]. Briefly, a crude extract was obtained from freeze-dried plant samples by stirring overnight with a mixture of methanol, water, and formic acid (80:19.9:0.1 v/v/v). The extraction was repeated five times, and the obtained crude extracts were collected. The free forms of phenolic acids and flavonoids were then isolated with diethyl ether. Next, after the free forms were isolated, esters present in the extracts were hydrolyzed with 4 M NaOH. Subsequently, glycosides present in the extracts were hydrolyzed in the residues with 6 M HCl. After each step, the released compounds were isolated with diethyl ether, and the ether was evaporated to dryness under a stream of nitrogen. The free compounds and compounds released from bound forms were dissolved in methanol, centrifuged and subjected to analysis on an HPLC-MS/MS system (QTRAP 5500 ion trap mass spectrometer, AB SCIEX, Canada) equipped with a HALO C18 column (2.7 μ m particles, 0.5 \times 50 mm, Eksigent, Canada) at 45°C, with a flow rate of 0.015 mL min⁻¹. The gradient of the elution solvents A (water/formic acid; 99.05/0.95; v/v) and B (acetonitrile/formic acid, 99.05/0.95, v/v) was as follows: 5% B for 0.1 min, 5–90% B in 1.9 min, 90% B for 0.5 min, 90–5% B in 0.2 min, and 5% B for 0.3 min. The contents of phenolic compounds obtained by acid and alkaline hydrolysis were presented as a total, *i.e.*, the sum of their free, ester, and glycosidic forms.

Measurements were subjected to analysis of variance (ANOVA) using STATISTICA 13.1 (StatSoft, Kraków, Poland), followed by Duncan's multiple range test. For statistical analysis, three replicates were used (each replicate consisted of 30 measurements for histological analysis and 20 or 40 mg of plant materials for analyses of phytohormones and metabolites). *P* values of <0.05 were considered to be statistically significant for means.

RESULTS

Ten days after treatment, NPA (5% w/w, in lanolin) significantly induced swelling of the *S. canadensis* stem at the site of its application. This swelling gradually diminished at a distance of about 10 cm from the application site (Fig. 1 and 2). The symptoms of stem swelling appeared as early as four days after applying NPA. NPA did not affect the increase in stem length compared to control plants during the flowering stage, but some growth occurred at about 70 cm above the treatment site (Fig. 1). Moreover, it was noted that the use of NPA at a concentration of 0.5% induced stem swelling to a slightly lesser degree (data not shown).

Microscopic analyses with the transverse and longitudinal sections through the stem of *S. canadensis* revealed significant differences in stem architecture between the sites treated with lanolin (control) and those treated with NPA (Fig. 3–5). The main distinction between the control and NPA-treated sites was the structure of the vascular bundles. The cambial zone in the swelling part of the stem was twice as wide compared to control plants. Both anticlinal and periclinal divisions of vascular cambium cells were observed in the NPA-treated part of the stem. Cambial cell proliferation resulted in the increased production of the secondary xylem, which led to significant thickening of vascular bundles compared to control (Fig. 3). A significant increase in the width of the cambial and xylem zones was also observed in the longitudinal section (Fig. 4). Additionally, the size of pith parenchymatous cells and the pith diameter increased at the site of stem swelling. However, the application of NPA had little or no effect on the size of cortex cells in the swelling part of the stem. Furthermore, there were no differences in the anatomical structure of the shoots below the NPA treatment site compared to the control (Fig. 5).

In the stem of *S. canadensis* the following auxins and their related compounds were identified: indole-3-acetic acid (IAA), indole-3-acetamide (IAM), indole-3-acetonitrile (IAN), 2-oxoindole-3-acetic acid (OxIAA), indole-3-carboxylic acid (ICA), indole-3-acetyl-aspartic acid (IAAsp), indole-3-acetyl-glutamic acid (IAGlu) and indole-3-propionic acid (IPA) (Fig. 6, Tab. 1a). In NPA-treated plants, the content of IAA in the stem was almost three times higher than

in the stem of control. The highest contents of IAA were found in the swelling part of the stem and the lowest below the treatment. In the swelling part of the stem, the contents of IAA metabolites, i.e. IPA, ICA and IAAsp, were also higher. However, little effect was shown on the contents of IAM, IAN, OxIAA and IAGlu in the stem swelling under NPA, as well as above and below the application site.

Five cytokinins were found in the tissue of *S. canadensis* stems: *trans*-zeatin (tZ), *cis*-zeatin (cZ), kinetin (K), *trans*-zeatin riboside (t-ZR), and *cis*-zeatin riboside (c-ZR) (Fig. 6, Tab. 1a). The t-ZR content increased significantly in the area of NPA-induced stem swelling but to a lesser extent in parts of the stem above and below treatment. The contents of other cytokinins, such as tZ, cZ, K, and c-ZR, were similar in the NPA-treated stem parts compared to those in control plants.

NPA had no effect on jasmonic acid (JA) and N-jasmonyl-leucine (JA-Leu) levels in the stem compared to the control, but 12-oxo-phytodienoic acid (12-oxo-PDA) contents were slightly lower below the NPA application site (Tab. 1b). The contents of salicylic acid (SA), benzoic acid (BeA), abscisic acid (ABA), and abscisic acid-leucine (ABA-Leu) in NPA-treated stem tissues were almost the same as in control plants (Tab. 1ab).

The following gibberellins were found in the stem of *S. canadensis*: GA₁, GA₃, GA₄, GA₅, GA₆, GA₇, GA₈, GA₉, GA₁₅, GA₁₉, GA₂₀, GA₄₄, and GA₅₃, although NPA application had little effect on their content (Tab. 1b).

NPA had a significant effect on the contents of most polar metabolites in the stem of *S. canadensis* at the site of its application, as well as above and below it. The content of monosaccharides, i.e., glucose, fructose and galactose, was substantially higher at the NPA application site on the stem and above it, compared to the content below the application site and in the control plants (Fig. 7). However, sucrose levels in the swelling part of the stem, as well as above and below the NPA application, were almost the same.

The NPA-induced swelling part of the stem and the area above this site showed a significant increase in the contents of *myo*-inositol and *D-chiro*-inositol compared to the control (Fig. 7). Levels of intermediate metabolites of the tricarboxylic acid cycle, such as

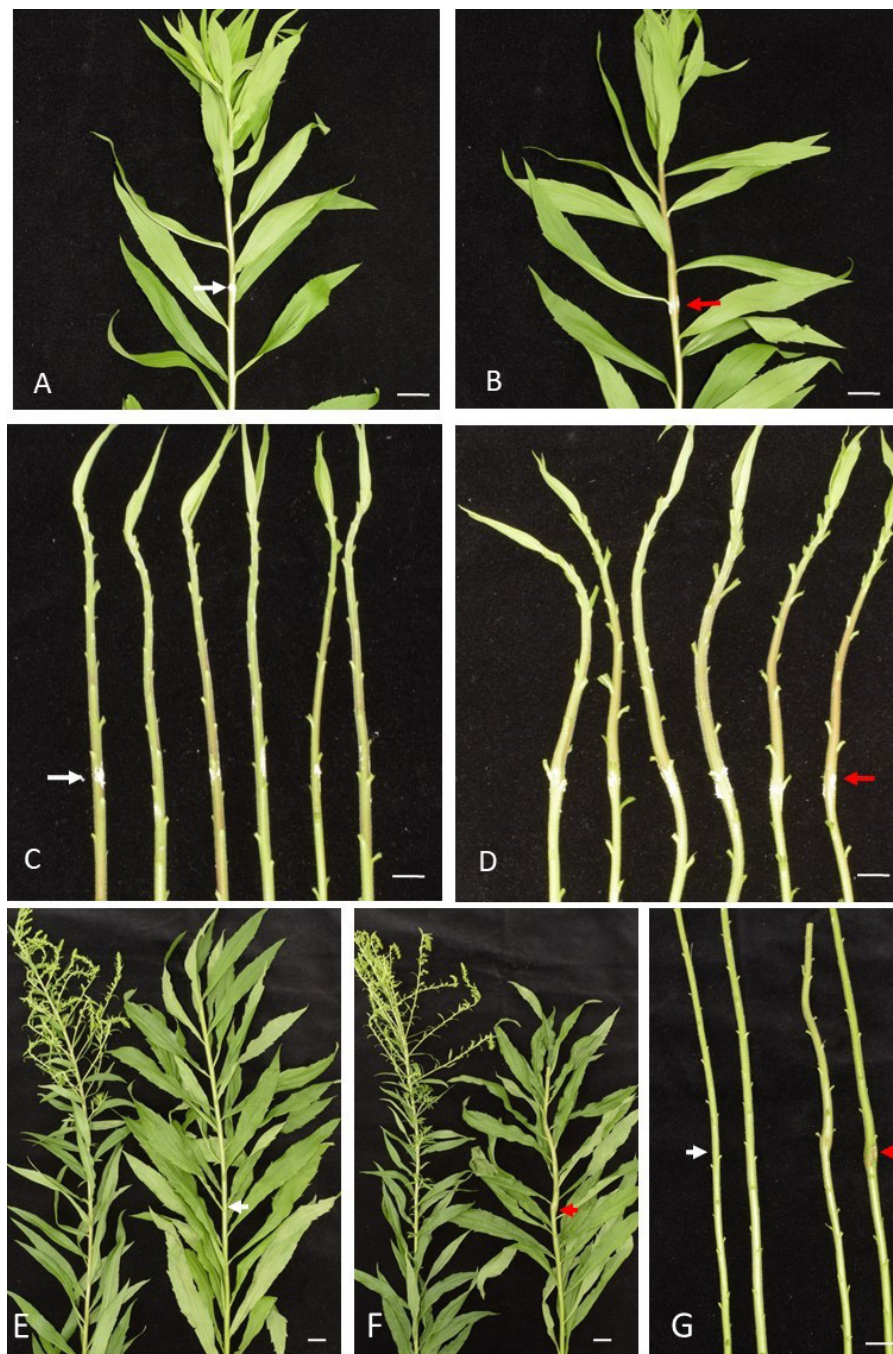


Fig. 1. NPA-induced stem swelling in *Solidago canadensis*. (A–D) After ten days of treatment on 4 June 2022. (A) Control (lanolin), the upper part of the stem with leaves, (B) NPA treatment, the upper part of the stem with leaves, (C) Control (lanolin), the upper part of the stem after excision of leaves, (D) NPA treatment, the upper part of the stem after excision of leaves. (E–G) The growth of *Solidago canadensis* stem treated with NPA on 11 June 2022. Pictures were taken on August 17, 2022. (E) Control (lanolin), the upper part of the stem with leaves, (F) NPA treatment, the upper part of the stem with leaves, (G) Control (lanolin), (left) and NPA treatment (right) after excision of leaves. White arrows indicate the place treated with lanolin only. Red arrows indicate the area of NPA application. Bars represent 10 mm

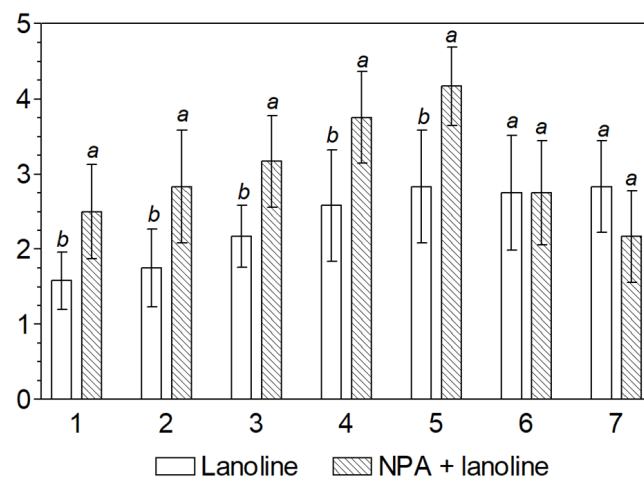


Fig. 2. Effect of NPA on stem diameter (means \pm SD, mm) in *Solidago canadensis*. Description of results: 1 – diameter 4 cm above treatment; 2 – diameter 3 cm above treatment; 3 – diameter 2 cm above treatment; 4 – diameter 1 cm above treatment; 5 – diameter at the treatment site; 6 – diameter 1 cm below treatment; 7 – diameter 2 cm below treatment. Different letters indicate statistical differences in Duncan’s multiple range test at $p \leq 0.05$ after ANOVA

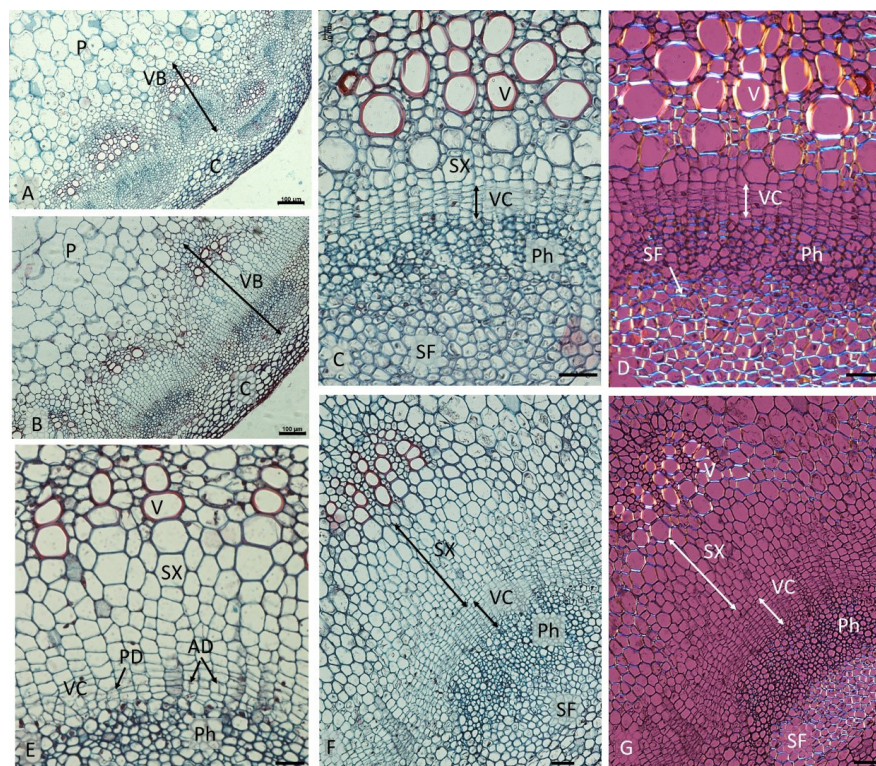


Fig. 3. The effect of NPA on conspicuous local stem swelling. Cross-section of the stem treated with lanolin (control) (A) and with NPA (B) in a light microscope (LM). A–B bars represent 100 μ m. Details of vas-cular tissue on the cross-section of a control stem in LM (C) and in the light microscopic image under polarized light (LMP) (D) and of stem swelling induced by the application of NPA in LM (F) and LMP (G). (E) Anticlinal and periclinal cell divisions in cambium. C–G bars represent 25 μ m. AD: anticlinal division; C: cortex; P: pith; PD: periclinal division; Ph: phloem; SF: vascular fibers; SX: secondary xylem; V: vessel; VB: vascular bundle; VC: vascular cambium

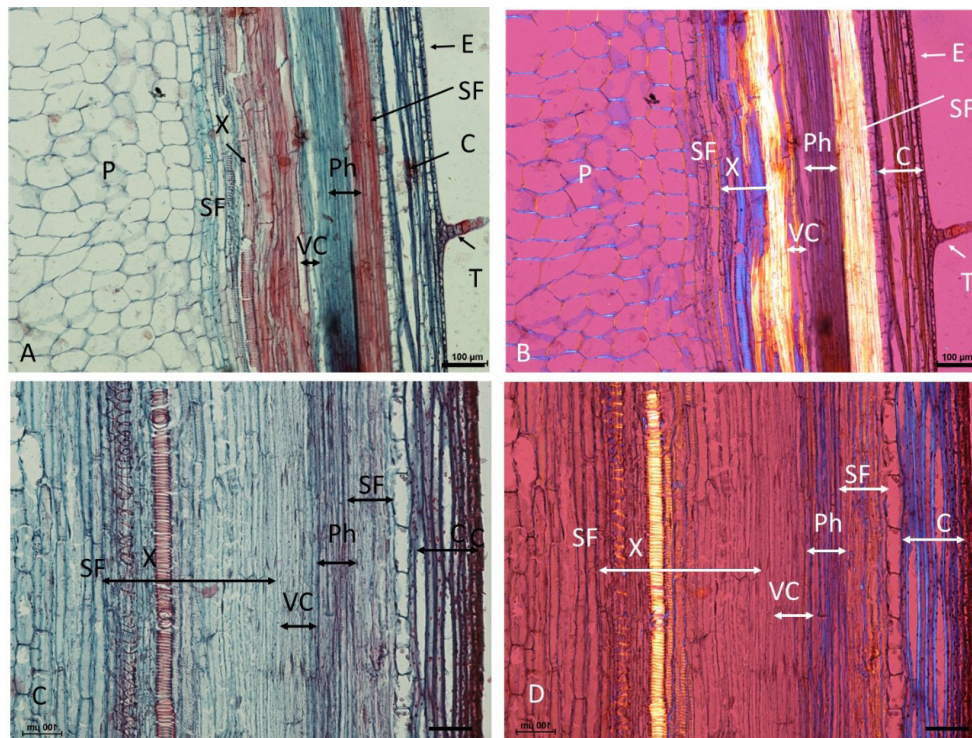


Fig. 4. Effect of NPA on conspicuous local stem swelling. Details of vascular tissue on the longitudinal section of the control stem in LM (A) and in LMP (B), and stem swelling induced by the application of NPA in LM (C) and LMP (D). A–D bars represent 100 μ m. CO: collenchyma; C: cortex; E: epidermis; P: pith; Ph: phloem; SF: vascular fibers; T: glandular trichome; VB: vascular bundle; VC: vascular cambium; X: xylem

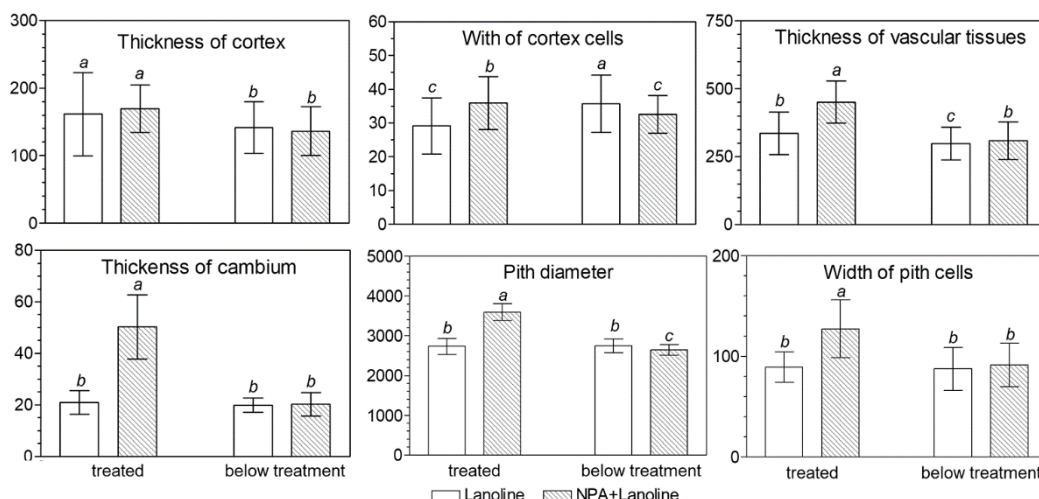


Fig. 5. Morphological and anatomical indices of *Solidago canadensis* stems at and below the NPA application site compared to the control (lanolin). Means within each graph followed by the same letter were not significantly different using Duncan's multiple range test at $p < 0.05$ after ANOVA

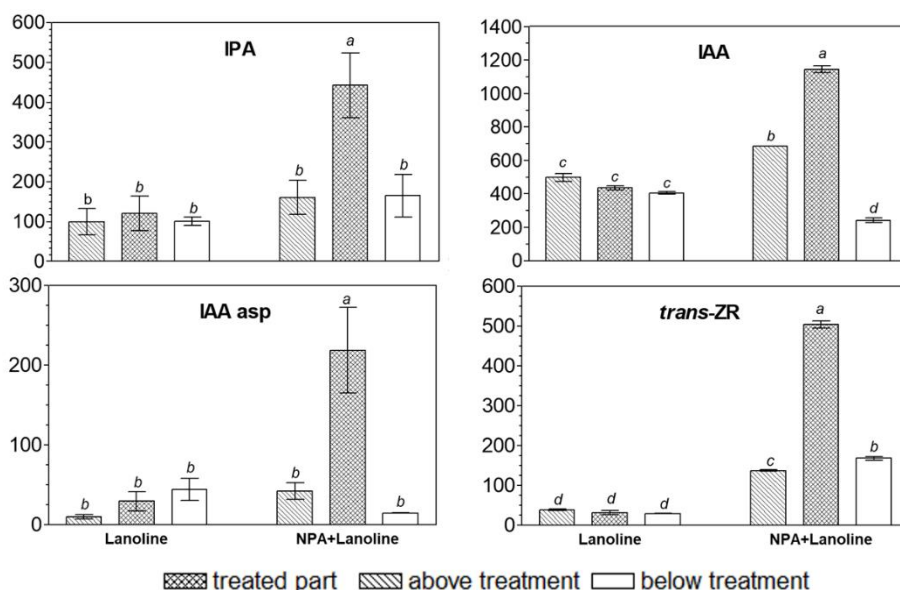


Fig. 6. Phytohormones content (means \pm SD, ng g^{-1} DW) at the site of NPA treatment (swelling part of the stem), as well as above and below the application site on the stem of *Solidago canadensis*. Different letters indicate statistical differences in Duncan's multiple range test at $p \leq 0.05$ after ANOVA

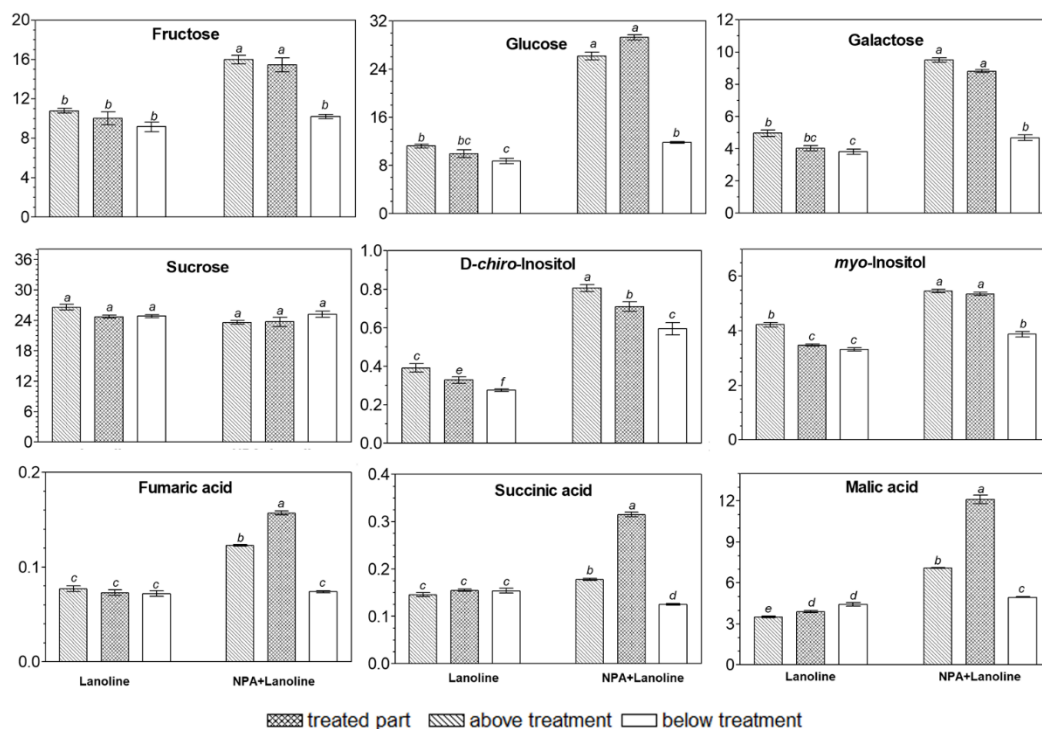


Fig. 7. Polar metabolites content (means \pm SD, mg g^{-1} DW) at the site of NPA treatment (swelling part of the stem), as well as above and below the application site on the stem of *Solidago canadensis*. Different letters indicate statistical differences in Duncan's multiple range test at $p \leq 0.05$ after ANOVA

Table 1a. Effect of NPA on levels of phytohormones (ng g⁻¹ DW) at the site of NPA application, as well as below and above it, in the stem of *Solidago canadensis*. Different letters indicate statistical differences in Duncan's multiple range test at *p* < 0.05 after ANOVA

Plant hormone	Control (lanolin)			NPA treatment		
	Above	Treated area	Below	Above	Treated area	Below
IAM	15.5 ±2.5 a	15.4 ±3.0 a	15.4 ±4.0 a	13.5 ±1.3 a	30.0 ±5.3 b	24.6 ±6.6 b
IAN	66.8 ±4.7 a	72.6 ±8.0 a	70.3 ±9.2 a	68.3 ±6.0 a	66.5 ±8.5 a	71.0 ±7.0 a
IPA	99.8 ±33.2 a	120.5 ±54.1 a	100.5 ±10.5 a	160.5 ±42.9 a	442.3 ±101.6 b	165.0 ±52.9 a
IAA	498.7 ±23.9 d	437.5 ±12.7 c	406.0 ± 6.8 b	684.9 ±1.7 e	1145.0 ±20.4 f	242.1 ±14.3 a
ICA	3948.3 ±78.8 d	2581.6 ±136.4 b	2116.7 ±170.6 a	5645. ±334.6 e	6081.8 ±174.3 f	3290.2 ±333.3 c
OxIAA	160.8 ±29.7 a	179.7 ±8.0 a	152.3 ±12.6 a	223.5 ±2.2 b	208.9 ±12.2 b	216.3 ±17.9 b
IAAsp	9.9 ±2.6 a	29.2 ±12.3 a	43.8 ±44.0 a	41.8 ±10.5 a	218.6 ±53.8 b	14.6 ±0.6 a
IAGlu	12.6 ±2.8 a	36.9 ±19.6 bc	14.3 ±7.4 a	23.7 ±7.6 abc	40.2 ±12.7 c	18.2 ±4.8 ab
tZ	6.3 ±0.0 b	5.8 ±0.14 a	5.8 ±0.24 a	6.4 ±0.15 b	7.3 ±0.4 c	6.5 ±0.1 b
cZ	0.51 ±0.0 ab	0.57 ±0.05 b	0.56 ±0.09 b	0.48 ±0.04 ab	0.45 ±0.0 a	0.48 ±0.0 ab
K	26.3 ±0.5 a	27.7 ±1.9 a	28.8 ±2.9 a	28.9 ±1.8 a	26.3 ±0.6 a	27.5 ±0.9 a
t-ZR	38.1 ±2.0 a	31.1 ±5.4 a	29.1 ±0.5 a	137.4 ±1.6 b	503.8 ±9.2 d	167.9 ±4.3 c
c-ZR	71.4 ±0.7 c	91.6 ±2.2 d	70.0 ±1.6 c	37.8 ±1.1 b	26.7 ±0.2 a	70.0 ±1.5 c
SA	5225.5 ±1132.8 ab	4681.0 ±668.4 a	4535.8 ±90.4 a	4630.3 ±314.1 a	5982.3 ±850.2 b	5836.7 ±366.7 ab
BeA	9380.4 ±1096.6 a	8491.8 ±300.2 a	8651.2 ±1414.4 a	8153.5 ±2480.2 a	9029.4 ±747.9 a	10496.4 ±1441.2 a
ABA	1496.7 ±126.6 d	1391.9 ±35.0 c	1295.4 ±94.4 bc	1351.7 ±21.8 c	1223.5 ±32.8 b	988.6 ±23.5 a
ABA-Leu	3.3 ±0.4 a	7.1 ±2.7 b	5.2 ±2.96 ab	5.6 ±0.44 ab	7.0 ±1.5 b	5.3 ±1.7 ab

Table 1b. Effect of NPA on levels of phytohormones (ng g⁻¹ DW) at the NPA application site, as well as below and above it, in the stem of *Solidago canadensis*. Different letters indicate statistical differences in Duncan's multiple range test at *p* < 0.05 after ANOVA

Plant hormone	Control (lanolin)			NPA treatment		
	Above	Treated area	Below	Above	Treated area	Below
JA	15008.1 ±891.6 ab	12567.2 ±843.9 a	12795.7 ±1568.4 a	15454.8 ±1142.8 ab	13063.5 ±1991.1 a	16880.0 ±2936.6 b
JA-Leu	935.8 ±28.8 c	620.7 ±44.0 a	642.0 ±60.2 a	888.2 ±64.1 bc	729.0 ±94.3 ab	957.8 ±203.6 c
12-oxo-PDA	1031.2 ±121.5 b	917.3 ±99.0 b	1040.5 ±20.4 b	901.3 ±11.7 b	615.0 ±83.9 a	1349.6 ±158.0 c
GA ₁	20.0 ±3.8 a	20.0 ±2.2 a	20.0 ±1.4 a	19.7 ±2.6 a	17.5 ±0.7 a	19.8 ±1.1 a
GA ₃	75.0 ±58.8 a	62.8 ±18.9 a	98.8 ±77.7 a	119.7 ±98.9 a	109.6 ±72.2 a	121.7 ±63.7 a
GA ₄	91.4 ±21.7 b	81.1 ±14.8 ab	68.3 ±11.5 ab	75.0 ±9.9 ab	62.5 ±12.3 a	89.4 ±11.2 ab
GA ₅	43.9 ±30.8 a	47.6 ±36.0 a	77.4 ±39.6 a	34.8 ±12.7 a	36.1 ±19.3 a	53.0 ±31.1 a
GA ₆	180.4 ±18.6 ab	198.6 ±19.7 b	119.7 ±77.5 ab	158.0 ±46.3 ab	102.3 ±63.6 a	143.8 ±12.8 ab
GA ₇	91.5 ±117.0 a	72.2 ±24.1 a	64.3 ±30.8 a	107.0 ±23.3 a	134.9 ±101.1 a	84.0 ±86.3 a
GA ₈	63.5 ±25.7 a	104.9 ±27.0 a	80.4 ±68.9 a	52.5 ±16.7 a	103.4 ±52.7 a	63.9 ±37.8 a
GA ₉	47.0 ±1.4 a	43.2 ±4.1 a	46.6 ±3.2 a	46.3 ±5.8 a	40.9 ±9.8 a	42.4 ±4.7 a
GA ₁₅	20.6 ±1.1 cd	21.6 ±0.76 d	19.4 ±0.37 bc	16.9 ±0.46 a	16.3 ±0.6 a	18.1 ±0.3 b
GA ₁₉	103.1 ±5.7 a	109.7 ±3.4 ab	117.8 ±9.6 ab	93.8 ±19.9 a	129.2 ±5.1 b	112.2 ±19.0 ab
GA ₂₀	42.3 ±20.5 a	47.4 ±9.7 a	86.4 ±29.9 b	25.8 ±2.4 a	35.1 ±29.1 a	15.5 ±3.5 a
GA ₄₄	0.50 ±0.2 a	0.22 ±0.12 a	0.64 ±0.38 a	0.25 ±0.19 a	0.75 ±0.4 a	0.38 ±0.5 a
GA ₅₃	57.2 ±26.3 ab	53.8 ±9.8 ab	36.3 ±18.3 a	37.9 ±14.5 a	87.4 ±53.0 ab	102.4 ±22.1 b

Table 2. Content of amino acids and other acids (means \pm SD, mg g⁻¹ DW) in *Solidago canadensis* stems treated with NPA and lanolin. Different letters in rows indicate statistical differences in Duncan's multiple range test at $p \leq 0.05$ after ANOVA

Analyzed compound	Control (lanolin)			NPA in lanolin		
	Above	Treated area	Below	Above	Treated area	Below
Alanine	0.121 \pm 0.008 ^b	0.114 \pm 0.006 ^b	0.114 \pm 0.012 ^b	0.146 \pm 0.013 ^b	0.206 \pm 0.011 ^a	0.131 \pm 0.017 ^b
Serine	0.048 \pm 0.005 ^a	0.046 \pm 0.003 ^a	0.041 \pm 0.003 ^a	0.038 \pm 0.002 ^a	0.052 \pm 0.004 ^a	0.049 \pm 0.005 ^a
Threonine	0.037 \pm 0.002 ^a	0.039 \pm 0.003 ^a	0.041 \pm 0.003 ^a	0.021 \pm 0.001 ^b	0.021 \pm 0.002 ^b	0.035 \pm 0.002 ^a
Aspartic acid	0.042 \pm 0.001 ^a	0.043 \pm 0.001 ^a	0.045 \pm 0.002 ^a	0.042 \pm 0.002 ^a	0.044 \pm 0.001 ^a	0.041 \pm 0.003 ^a
Total amino acids	0.248 \pm 0.013 ^b	0.242 \pm 0.011 ^b	0.241 \pm 0.012 ^b	0.247 \pm 0.011 ^b	0.323 \pm 0.011 ^a	0.256 \pm 0.010 ^b
Phosphoric acid	8.106 \pm 0.132 ^b	8.089 \pm 0.160 ^b	7.924 \pm 0.229 ^b	8.075 \pm 0.063 ^b	7.806 \pm 0.206 ^b	9.006 \pm 0.081 ^a
Ribonic acid	0.414 \pm 0.014 ^a	0.367 \pm 0.017 ^{ab}	0.329 \pm 0.004 ^b	0.433 \pm 0.010 ^a	0.392 \pm 0.010 ^a	0.385 \pm 0.028 ^{ab}
Glyceric acid	0.046 \pm 0.001 ^b	0.043 \pm 0.002 ^b	0.042 \pm 0.002 ^b	0.052 \pm 0.001 ^a	0.052 \pm 0.001 ^a	0.043 \pm 0.002 ^b
γ -Aminobutyric acid (GABA)	0.101 \pm 0.004 ^c	0.108 \pm 0.002 ^c	0.116 \pm 0.004 ^{bc}	0.136 \pm 0.002 ^a	0.131 \pm 0.004 ^{ab}	0.136 \pm 0.001 ^a
Lactic acid	0.157 \pm 0.015 ^c	0.134 \pm 0.018 ^c	0.117 \pm 0.011 ^{cd}	0.228 \pm 0.08 ^b	0.318 \pm 0.031 ^a	0.088 \pm 0.015 ^d
Oxalic acid	0.205 \pm 0.016 ^a	0.210 \pm 0.013 ^a	0.208 \pm 0.014 ^a	0.189 \pm 0.028 ^a	0.224 \pm 0.027 ^a	0.200 \pm 0.008 ^a
Shikimic acid	1.023 \pm 0.020 ^b	0.779 \pm 0.019 ^d	0.673 \pm 0.021 ^c	1.226 \pm 0.006 ^a	0.870 \pm 0.011 ^c	0.980 \pm 0.017 ^b
Quinic acid*	0.711 \pm 0.017 ^a	0.606 \pm 0.010 ^c	0.562 \pm 0.015 ^{cd}	0.647 \pm 0.005 ^b	0.551 \pm 0.009 ^d	0.544 \pm 0.010 ^d

*This compound was identified based on the NIST database; due to the lack of a standard, its results were calculated using an internal standard (ribitol)

Table 3. Content of phenolic acids and flavonoids (means \pm SD, mg g⁻¹ DW) in *Solidago canadensis* stems treated with NPA and lanolin. Different letters in rows indicate statistical differences in Duncan's multiple range test at $p \leq 0.05$ after ANOVA

Analyzed compound	Control (lanolin)			NPA in lanolin		
	Above	Treated area	Below	Above	Treated area	Below
Phenolic acids						
3-Hydroxybenzoic acid	1.48 \pm 0.10 ^b	1.82 \pm 0.13 ^{ab}	1.68 \pm 0.06 ^{ab}	2.04 \pm 0.09 ^a	1.60 \pm 0.09 ^{ab}	1.92 \pm 0.08 ^{ab}
Ferulic acid	0.15 \pm 0.02 ^a	0.13 \pm 0.01 ^a	0.13 \pm 0.01 ^a	0.22 \pm 0.04 ^a	0.16 \pm 0.02 ^a	0.13 \pm 0.02 ^a
Caffeic acid	0.19 \pm 0.03 ^b	0.24 \pm 0.03 ^{ab}	0.15 \pm 0.03 ^b	0.45 \pm 0.03 ^a	0.35 \pm 0.03 ^a	0.18 \pm 0.03 ^b
5- <i>O</i> -Caffeoyl-quinic acid (<i>neo</i> -Chlorogenic acid)	1.88 \pm 0.02 ^b	1.14 \pm 0.05 ^d	1.71 \pm 0.02 ^c	2.23 \pm 0.08 ^a	1.52 \pm 0.06 ^c	0.86 \pm 0.03 ^c
3- <i>O</i> -Caffeoyl-quinic acid (Chlorogenic acid)	0.14 \pm 0.01 ^{ab}	0.10 \pm 0.01 ^{bc}	0.14 \pm 0.01 ^{ab}	0.18 \pm 0.02 ^a	0.12 \pm 0.01 ^{abc}	0.07 \pm 0.01 ^c
4-Feruloyl-quinic acid	0.26 \pm 0.01 ^a	0.14 \pm 0.01 ^d	0.22 \pm 0.01 ^b	0.31 \pm 0.01 ^a	0.22 \pm 0.02 ^{bc}	0.16 \pm 0.01 ^{cd}
<i>di</i> -Caffeoyl-quinic acid	1.39 \pm 0.06 ^b	0.98 \pm 0.06 ^c	1.44 \pm 0.05 ^b	1.94 \pm 0.06 ^a	1.33 \pm 0.03 ^b	0.62 \pm 0.01 ^d
Total caffeic acid and its derivatives	3.86 \pm 0.14 ^b	2.60 \pm 0.16 ^c	3.66 \pm 0.12 ^b	5.11 \pm 0.20 ^a	3.54 \pm 0.15 ^b	1.89 \pm 0.09 ^d
Flavonoids						
Rutin	0.19 \pm 0.01 ^a	0.08 \pm 0.01 ^{bc}	0.15 \pm 0.02 ^{ab}	0.21 \pm 0.02 ^a	0.12 \pm 0.01 ^{bc}	0.07 \pm 0.01 ^c
Quercetin	0.06 \pm 0.01 ^a	0.02 \pm 0.01 ^a	0.04 \pm 0.01 ^a	0.04 \pm 0.01 ^a	0.02 \pm 0.01 ^a	0.02 \pm 0.01 ^a
<i>Iso</i> -rhamnetin	0.08 \pm 0.02 ^a	0.03 \pm 0.01 ^a	0.07 \pm 0.01 ^a	0.05 \pm 0.01 ^a	0.03 \pm 0.01 ^a	0.02 \pm 0.01 ^a

malic acid, succinic acid, and fumaric acid, also increased in the swelling part of the stem compared to the area below the treatment and the stems of control plants (Fig. 7).

The contents of shikimic and quinic acids in the stem treated with NPA did not change, but they increased in the stem above the application site (Tab. 2). Among the amino acids present in measurable contents in the swelling part of the *S. canadensis* stem, as well as below and above it, the applied NPA increased alanine, decreased threonine, and did not affect the levels of serine, aspartic acid, and γ -aminobutyric acid (GABA). On the other hand, lactic acid content was about 3.5 times higher at the NPA application site and about 1.5 times higher above the site than below it.

Analysis of phenolic compounds present in *S. canadensis* stem tissues was carried out using the LC-ESI-MS/MS method. This analysis revealed the presence of several phenolic acids and a small amount of flavonoids (Tab. 3). Among them, 5-O-caffeoyl-quinic acid (neo-chlorogenic acid), di-caffeoyl-quinic acid, and 3-hydroxybenzoic acid were the main compounds quantitatively. The application of NPA increased the contents of neo-chlorogenic acid, di-caffeoyl-quinic acid, and total quinic acid derivatives at and above the swelling part of the stem (Tab. 3). The flavonoid content was relatively low, and the application of NPA had a minor effect on it.

DISCUSSION

As mentioned in the Introduction, *S. canadensis* is an extremely effective invasive weed native to North America [Baranová et al. 2022]. From an ecological point of view, the biomass of this weed should be reduced as efficiently as possible [Bielecka et al. 2017]. In the present study, the effect of NPA, an auxin herbicide that interferes with the growth and development of *S. canadensis*, was comprehensively evaluated. The study focused on changes in the histological structure of plant stems, as well as an analysis of phytohormone content and various primary and secondary metabolites to clarify the mode of action of NPA and its possible use in controlling the spread of this species.

The application of NPA to the stem of *S. canadensis* strongly induced swelling. The swelling was most

visible at the site of NPA application and gradually decreased to 10 cm below this site (Fig. 1 and 2). Microscopic analyses revealed that NPA substantially affected the histological structures of the stem, especially the size of cambial cells, by inducing the thickness of vascular bundles (Fig. 3, 4 and 5). Histological changes caused by NPA have been previously reported in tomato seedlings [Nongmaithem et al. 2020]. In this species, NPA inhibited root growth and stimulated hypocotyl elongation, and the epidermal cells were more elongated and narrower than control seedlings. Moreover, it was earlier found that applying NPA in lanolin to the shoots of *Pinus sylvestris* and the shoots of *Cryptomeria japonica* resulted in the formation of compression wood above the site of application [Yamaguchi et al. 1980, Sundberg et al. 1994]. The authors of these studies suggested that NPA-induced stem swelling is the result of enhancing auxin-dependent processes such as cambium activity and xylem production. Later, Little et al. [2002] showed that treatment of the *A. thaliana* inflorescence stems with NPA inhibited its radial development below the site of application, as well as the development of fascicular xylem, interfascicular xylem and interfascicular extrinsic fibers while increasing the production of fascicle xylem above the treatment site. Moreover, NPA-treated stem of *Populus tremula xalba* in a longitudinal strip formed aberrant vessels along the stem length that were short and narrow, as well as highly clustered and angular in cross-section. In contrast, the xylem formed on the untreated side of the stem contained typical vessels [Johnson et al. 2018]. A similar phenomenon of NPA-induced stem swelling occurred in *Bryophyllum calycinum* [Marasek-Ciolakowska et al. 2021]. Suer et al. [2011] also reported that NPA-induced auxin accumulation stimulated cambial activity in wild-type *A. thaliana* stems, but not in WOX4 mutants. These facts, together with the results of the current study, suggest that limiting the spread of *S. canadensis* in the environment is possible by using NPA to change histological structures, especially in the cambium zone.

To the knowledge of the authors of the current study, only a few reports are available on the occurrence of phytohormones and their role in the growth and development of *S. canadensis*. In the current study, phytohormones such as IAA and related compounds, cy-

tokinins, ABA, SA, JAs, and GAs were detected and analyzed at the site of NPA-induced swelling, as well as above and below this site. A total of 33 compounds of phytohormonal significance were identified and assayed in *S. canadensis* (Tab. 1ab). Therefore, this study is the first report containing the results of such a comprehensive analysis of phytohormones in this species. The results obtained indicate that NPA significantly increased the level of IAA in the swelling site of *S. canadensis* stem, to a lesser extent in the stem above the treatment but not below it (Fig. 6, Tab. 1). This could mean that NPA inhibits polar auxin transport in the stem of *S. canadensis*, although its more precise analysis using radioactively labelled IAA has not yet been carried out. Inhibition of polar auxin transport by NPA in the stem of *S. canadensis*, which the authors discovered, is a confirmation of several earlier studies on *A. thaliana* [Zhong and Ye 2001, Little et al. 2002, Suer et al. 2011, Yamaguchi et al. 1980]. The contents of IPA, ICA and IAAsp significantly increased only at the site of swelling in response to NPA, whereas the levels of IAM, IAN, OxIAA, and IAGlu remained unchanged (Fig. 6, Tab. 1). It should be indicated that the natural occurrence of IPA in plants is rare, and to date, little is known about its physiological significance [Ludwig-Müller 2020]. The occurrence of IPA was confirmed in seedlings of *Cucurbita pepo* [Segal and Wightman 1982] and in the stem of *Bryophyllum calycinum* [Dziurka et al. 2022]. Recently, the complete pathway of oxidative auxin inactivation has been established, and the role of auxin homeostasis in plant development has been determined [Hayashi et al. 2021]. Until now, it has been believed that IAA-amino acid conjugates, such as IAAsp and IAGlu, are not a form of auxin storage [Korasick et al. 2013]. However, Hayashi et al. [2021] discovered that IAAsp and IAGlu can serve as primary storage forms of IAA *in vivo* and can be converted back to IAA. These authors also demonstrated that IAA is not directly oxidized to OxIAA, but rather IAA is first converted to IAAsp and IAGlu, then to acetic acid 2-oxindole-3-aspartate (OxIAAsp) and 2-oxindole-3-acetic acid-glutamate (OxIAGlu). They are then hydrolyzed to OxIAA [Zhang and Peer 2017].

Among the five cytokinins found in the stem of *S. canadensis*, only t-ZR levels increased markedly above and below the site of NPA treatment (Fig. 6, Tab. 1). According to the authors' previous studies, NPA-

induced stem swelling in *Bryophyllum calycinum* was also accompanied by increased levels of auxins, cytokinins, JA, and 12-oxo-PDA at the treatment site [Marasek-Ciolakowska et al. 2021]. It seems that the interaction of auxins and cytokinins, which accumulated as a result of NPA treatment, is responsible for the stem swelling in both species. It was previously reported that NPA treatment mimicked cytokinin induction in the off-the-medium growth of the *Arabidopsis* root tip [Kushwah et al. 2011]. Hu et al. [2017] showed that NPA accelerated shoot organogenesis in the epicotyl of citrus explants, independent of its involvement in auxin transport, and found that the effect of NPA on shoot organogenesis was dependent on cytokinin. Additionally, it was recently reported that NPA, in interaction with cytokinins, had a stimulating effect on the organogenesis of shoots from epicotyl explants of *Cannabis sativa* [Dreger and Szalata 2022]. Previously, the combined effect of NPA and cytokinins on changes in leaf development in tobacco was also demonstrated [Strabala et al. 1996]. Recently, Gong et al. [2022] showed that NPA-induced inhibition of soybean leaf growth was related to a decrease in the number of cells at the early stages of leaf expansion, as well as an increase in auxin levels and a decrease in cytokinins.

The application of NPA to the stem of *S. canadensis* induced a stress response in the form of stem swelling, which was accompanied by increases in auxin and cytokinin levels. The swelling phenomenon also occurred to a lesser degree above the treatment site. Thus, the swelling area of the stem causes the accumulation of auxins and cytokinins, probably by inhibiting their polar transport. It is interesting to note that Abreu et al. [2020] found that IAA and cytokinins accumulated in the cambial region of wood-forming tissue in *Populus tremula*. Those authors suggest that the mentioned phytohormones play an important role in controlling cell division and their positioning in the cambial region. They also found that sucrose levels declined rapidly from the cambial tissue toward the expansion zone, while the glucose and fructose levels gradually increased, reaching a maximum in the zone of secondary wall formation [Abreu et al. 2020].

Several studies have demonstrated cytokinin's role in controlling the cell division rate within the cambium [Matsumoto-Kitano et al. 2008, Immanen et al.

2016]. Recently, Sergeeva et al. [2021] showed that the application of benzylaminopurine to the cambium of *Arabis alpina* increased its activity, resulting in the formation of secondary phloem parenchyma in the proximal perennial zone and, to a lesser extent, in the distal annual zone.

Thus, the NPA-induced process of stem swelling in *S. canadensis*, together with the substantial accumulation of the auxin and cytokinins, suggests that these changes mimic the processes during cambium activity in plants in natural conditions. It has been shown that these phytohormones play a key role during the development of secondary vascular tissue [Zhang et al. 2014]. According to Chen et al. [2019], auxin and cytokinin interact to regulate secondary vascular tissue regeneration in the hybrid aspen (*Populus tremula* × *Populus tremuloides*). This is because auxin and cytokinin signaling genes interact to facilitate the regeneration of secondary vascular tissue. These findings, combined with the conclusions of the present study, strongly suggest that NPA may be a limiting factor in the invasion of *S. canadensis*, causing the formation of an abnormal structure such as stem swelling. This phenomenon was accompanied by the inhibition of polar auxin transport, leading to accumulation of auxin as well as an increase in tZR levels.

Auxins, depending on their levels, stimulate the mobilization of carbohydrates in source tissues and increase their transport toward the sink organs, thus regulating sink strength [Lemoine et al. 2013]. Roots and young leaves are major sinks during the early developmental stages, whereas tubers, fruit, and seeds become major sinks during the development of reproductive organs. Various environmental factors, such as water stress, salt stress, mineral deficiency, light intensity, temperature, soil contaminants, pathogens, parasites, etc., affect plant growth and development by disrupting the source/sink relationship [Lemoine et al. 2013]. The swollen part of *S. canadensis* stem, resulting from NPA application, appears to be a physiological sink, while the shoot above the application site serves as a source of various metabolites necessary for tissue growth in the swollen stem region. This is likely the main reason for the increased levels of monosaccharides (glucose, fructose, galactose), TCA acids (malic, succinic, and fumaric), cyclitols, and quinic acid derivatives at the site of NPA application. The mar-

kedly increased acid content may have resulted from increased respiration, but it could also have lowered the pH in this part of the stem, potentially affecting the activity of enzymes involved in the swelling phenomenon. The results obtained in the current study seem to confirm previous findings suggesting that the increase in the content of TCA acids in tomato seedlings after NPA treatment may be related to the regulation of this cycle [Nongmaithem et al. 2020]. The demonstrated increased levels of monosaccharides, cyclitols, and TCA-related acids above the part of the stem on which NPA was applied may indicate that NPA can also be transported acropetally, at least in part. Therefore, the levels of the mentioned metabolites were also elevated above the site of NPA application. It was previously found that inhibition of polar auxin transport by NPA in the roots of *Catsetum fimbriatum* reduced the level of IAA in leaves and pseudobulbs and caused significant accumulation of saccharides in pseudobulbs and organic acids in leaves. This indicates that auxin flow from roots to shoots is involved in carbon partitioning in the aerial organs of this plant [Oliveira et al. 2019]. IAA regulates sink strength by controlling the import of carbohydrates into sink organs, which affect plant growth and developmental responses [Mishra et al. 2021]. Close interactions between carbohydrates and auxin play major roles in various aspects of plant development, such as cell division and expansion [Hartig and Beck 2006, Wang and Ruan 2013]. Carbohydrates play a basic role as carbon and energy sources, as signaling molecules to regulate gene expression, and in the modification of auxin homeostasis [Sairanen et al. 2012]. Cytokinins, in interaction with auxins, are also involved in the regulation of the source/sink relationship [McIntyre et al. 2021].

Ahkami et al. [2013] showed that the application of NPA to the shoots of *Petunia hybrida* seedlings inhibited adventitious root formation. As a result, glucose and fructose contents in the stem base of these seedlings were three times higher, and sucrose contents were 1.5 times higher compared to untreated seedlings. These authors consider that the higher levels of sugars in NPA-treated seedlings were due to lower carbohydrate utilization as a consequence of the strong inhibition of root formation. Similar to the current results, the content of total amino acids was similar in both non-treated and NPA-treated parts of

the stem [Ahkami et al. 2013]. In the tissue of tomato seedlings, NPA also increased the content of mono-saccharides, which contribute to cell wall expansion [Nongmaithem et al. 2020]. The results of the current study on *S. canadensis* seem to confirm this phenomenon.

Recently, Balasubramanian et al. [2023] conducted an excellent study on the effects of foliar treatment with auxins and NPA on the metabolism and transcriptome of leaf, stem, and root tissues in poplar seedlings. These authors found sugar alcohols (arabitol, ribitol, *myo*-inositol), carbohydrates from starch metabolism, and TCA/glyoxylate cycle intermediates as key molecular factors in describing these effects. Metabolomic analysis revealed differences in leaf, stem, and root tissues between auxin and NPA treatments compared to controls.

CONCLUSIONS

The application of N-1-naphthylphthalamic acid (NPA), acting as an inhibitor of polar auxin transport, on the stem of *Solidago canadensis* resulted in the abnormal radial growth of the stem, changes in histological structure, and alterations in endogenous levels of phytohormones and polar metabolites. NPA application induced IAA accumulation and disturbed the IAA metabolism. In addition, NPA distinctly increased the endogenous trans-zeatin riboside level. The levels of glucose, fructose, galactose, malic acid, succinic acid, fumaric acid, cyclitols, and quinic acid derivatives increased significantly in the NPA-induced swelling stems.

Thus, the interaction of accumulated auxins and cytokinins seems to be responsible for the abnormal structure, which is the swelling of the *S. canadensis* stem, and likely affected the regulation of sink strength by controlling the transport of carbohydrates and other metabolites. However, sucrose contents in both the swelling part of the stem and above and below the NPA application site were almost the same, suggesting that its transport was not inhibited in the NPA-treated stem of *S. canadensis*.

Comprehensive histological, phytohormonal and metabolic analyses in response to NPA-induced stem swelling in *S. canadensis* indicate a general and multi-directional mechanism of action of NPA on this plant.

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